

## IN VIVO INSECT MODEL SYSTEM FOR TYPE-2 DIABETES

Technical Field

5 The present invention concerns a tool for the investigation of diseases, in particular type-2 diabetes. The invention especially concerns an in vivo model that enables e.g. the screening of compounds suitable for therapy and diagnosis.

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Background Art

Due to its homology with man, *Drosophila* might be a very useful in vivo model for the investigation of human diseases and for the drug  
15 screening, as long as the target to be investigated leads to an easily detectable change in the fly, such as size reduction.

Factors influencing the growth have already been extensively studied.

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Much of the knowledge about growth regulation stems from studies done using tissue culture cell lines. A large number of peptide growth factors have been identified that stimulate cell division and survival of cultured cells. These observations have led to the  
25 hypothesis that regulation of overall growth is controlled primarily by coordinating cell cycle progression and cell survival (Raff, 1996). Although during development, the regulation of the cell cycle is tightly coupled to morphogenetic events (Edgar et al,  
30 1994), several lines of evidence suggest that it may not be the primary determinant of growth regulation. Overall growth of an organ appears to be monitored by measuring total organ tissue volume and not by counting cell divisions. In mosaic wings consisting of haploid and  
35 diploid cells, haploid cells produce a normal sized compartment consisting of twice the number of cells (Santamaria, 1983). Moreover, if mitosis is blocked by

use of a temperature sensitive mutation in *cdc2* (Weigmann et al., 1997) or by overexpression of RBF, which is a negative regulator of the cell cycle (Neufeld et al., 1998), the result is normal sized compartments consisting of fewer but larger cells. Conversely, accelerating the cell cycle by overexpression of E2F, a positive cell cycle regulator produces more and smaller cells but does not alter clone size (Neufeld et al. 1998). Thus, changing the length of the cell cycle does not directly affect overall organ growth, which indicates that cellular growth can occur independently of cell cycle control.

In higher vertebrates, hormones and growth factors play an important role in the control of overall growth because they orchestrate cell growth, cell cycle, and cell survival. Reducing or increasing levels of growth hormone or of its mediators, IGF-1 and its receptor (IGFR), dramatically influence body and organ size (for review see Stewart and Rotwein, 1996). The control of cell survival can be an essential factor influencing overall growth (Raff, 1996).

Overall growth (and in some cases cell size) is also affected by the availability of nutrients. Many organisms have developed special survival strategies for periods of growth during low nutrition. Under inadequate nutritional conditions yeast cells, for example, reduce growth and divide at a smaller size (Thomas and Hall, 1997), whereas nematodes like *C. elegans* enter a diapause called the dauer stage (reviewed in Riddle, 1988; Cassada and Russel, 1975). When in this state, larvae arrest development in a sexually immature stage, alter their metabolism to increase the storage of fat, and live up to three times as long as under non-starved conditions (Cassada and Russel, 1975). Dauer formation in *C. elegans* is dependent on the cooperation of the insulin and TGF- $\beta$  signalling pathways (Kimura et al., 1997). Raising *Drosophila* under adverse food conditions also results in

the production of small flies with fewer and smaller cells (Robertson, 1959; Bryant and Simpson, 1984). Still, little is known in higher organisms about how growth is controlled at the cellular level. In *Drosophila*, a class of mutations known as *Minutes (M)* dominantly delay development, and in some cases result in reduced body size. Some of the *M* genes encode ribosomal proteins and are thought to slow down growth by reducing protein synthesis (Lindsley and Zimm, 1992). Partial loss of function mutations in the *Drosophila myc* gene, diminutive, cause a reduction in overall body size (Gallant et al., 1996).

There has also already been described a P element induced *Drosophila* mutation, *fs(2)4<sup>1</sup>*, as a female sterile, male fertile, mutation (Berg and Spradling, 1991). However, there is nothing disclosed about size differences or the place of the P-element induced mutation.

Thus, none of the documents of the state of the art suggests any applicability of such flies in the investigation of human diseases and/or for drug screening.

It is therefore still very much desired to get an in vivo model for such investigations.

#### Disclosure of the Invention

It has now surprisingly been found that the mutant strain, the phenotype of which was described by Berg and Spradling in 1991, after 7 years of storage lost its viability. The originally described female sterile phenotype could be restored by recombination. After recombination, two further phenotypes associated with the defect *fs(2)4<sup>1</sup>* could be observed: Homozygous animals are reduced in size and adult flies have increased lipid levels. Furthermore it was discovered that the mutation

is located in the gene coding for the homolog of the insulin receptor substrate (IRS 1-4).

Due to the observed reduction in the body size, the gene affected by the P element and the mutant animal, in particular the mutant *Drosophila*, with a size reducing defect in said gene, are named *chico* which means small boy in Spanish.

The observed alterations in the body size and other characteristics of the mutant make such mutant animals very useful tools in the investigation of interactions with and mutations of the insulin signaling pathway.

Thus, one object of the present invention was to provide a method for searching for compounds or mutations interacting directly or indirectly with the insulin signaling pathway, wherein a viable insect is treated with at least one compound or with at least one mutation generating means, and that the effect of such treatment on the body size and/or cell size and/or development time and/or lipid level is determined whereby alterations of the body size and/or cell size and/or development time and/or lipid level are detectable in at least part of the animal. Preferred animals for use in such method are animals with reduced *chico* function, e.g. with at least one nucleotide sequence being a *chico* mutation.

Another object of the present invention was to provide the *chico* gene and *chico* mutations, in particular size reducing *chico* mutations. *Chico* is of particular interest, since also homozygosity for *chico* causes only semi-lethality. Additionally, an overall delay in development has been observed. Homozygous *chico* animals eclose two or three days after their heterozygous siblings. Under noncrowded culture conditions, homozygous *chico* mutant females can produce few viable progeny lacking both maternal and zygotie *chico* function.

In the scope of the present invention, animal means insects, whereby preferred insects are flies and a preferred fly is *Drosophila*. Where in the scope of the further general description fly or *Drosophila* is mentioned, it has to be understood that - in not preferred embodiments of the present invention - also other animals of the above definition are encompassed in the respective disclosure.

The fact that insects with a mutation within the insulin signaling pathway show altered characteristics is e.g. shown in that mutant flies homozygous for *chico* are markedly reduced in size but survive. This shows that a total loss of *chico* activity is not lethal. Obviously, the insulin receptor (INR) pathway is thus that *chico* can - at least partially - be bypassed.

Due to this finding, *chico* mutant animals are not only suitable for drug screening, but also enable the search for possible further defects in the INR pathway or therewith interacting factors.

While for drug screening flies with two inactive *chico* mutants, one inactive *chico* mutant and one *chico* gene lost, or both *chico* genes lost are preferred, also flies with a reduced *chico* activity can be used as long as the mutant flies are sufficiently distinguishable from wild-type flies.

Such flies with reduced *chico* activity can either have two *chico* mutations with reduced activity both, one *chico* mutation with no and one *chico* mutation with reduced activity, or one *chico* totally lacking and one *chico* mutation with reduced activity.

Besides of drug screening, such *chico* mutant flies can also be used as sensitized model systems to find other key components in the INR pathway.

Although heterozygous *chico* flies with one functional *chico* are not easily distinguishable from wild-type flies, they are interesting models for finding

further key components in the INR pathway, whereby flies sensitized in *chico* and having a further non-lethal defect in another compound involved in the INR pathway, are again useful model systems for drug screening and search for further key components.

Flies comprising, besides *chico*, further mutations can be obtained by several methods known to the skilled person such as mutations induced by P element, chemical compounds, radiation etc. If a mutation of a known key component shall be introduced, this can furthermore be performed by destroying at least one respective wild-type gene and insertion of the desired mutation.

The preferred animal - as already mentioned above - is *Drosophila* due to the great knowledge on this fly, the high conservation of the genome and the fast reproduction. However, the high conservation makes also other insects suitable model systems, in particular other flies.

The applicability of *chico* mutations is not only particularly broad due to their specific characteristics, but also due to the fact that it is the first coupling element within the cell and thus situated at the very beginning of the cascade of components involved in the INR pathway.

The method of screening for compounds influencing the INR pathway involves *chico* mutant animals such that e.g. a single drug or a combination of two or more drugs is applied to the animal in as early development stage, e.g. to the larvae of said *chico* mutant flies and that at least one difference between treated and non treated animals in the juvenile or adult state, e.g. larvae or flies, respectively, is determined.

The time of the application is not critical. The application can be performed by injection, whereby a preferred method is injection into the egg, and a much preferred method is injection into the larvae. However,

for some applications, injection into the adult animal is also possible.

The determination of differences between treated and non-treated animals can be performed by measuring the weight, the cell size, the cell number or the lipid content per weight. The fact that *chico* affects not either cell size or cell number, but both is a very favorable characteristic if e.g. a second defect were present affecting either the number or the size only.

Thus, *chico* mutant flies, whether they have only *chico* mutations or furthermore another mutation in the INR pathway, are very useful tools for the screening of compounds improving the INR signalling pathway.

#### Brief Description of the Drawings

The invention will be better understood and objects other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such description makes reference to the annexed drawings, wherein:

Figure 1A shows the body size reduction of homozygous mutant animals at different development stages, whereby *Drosophila* with one wild-type *chico* (+) and one inactive *chico* allele (-) are marked as (+/-), *Drosophila* with two inactive *chico* alleles as (-/-) and control flies with two wild-type *chico* as (+/+), and whereby the *chico*<sup>-/-</sup> egg was derived from heterozygous *chico* females in which *chico*<sup>-/-</sup> germline clones had been generated. The *chico*<sup>1</sup> allele (P element insertion allele) was used for germ-line clones. In comparison, a heterozygous *chico* mutant egg (*chico*<sup>+/-</sup>) is shown. Homozygous *chico*<sup>-/-</sup> larvae, pupae and adults were derived from heterozygous flies carrying the *chico*<sup>2</sup> allele.

Figure 1B shows the body weight of individual flies (n = 20) whereby the weights of y w control flies

(+/+), heterozygous (+/-) and homozygous mutant (-/-) *chico* flies are shown.

Figure 2 represents comparison of the cell size distribution of heterozygous (+/-) and homozygous mutant (-/-) *chico* wing disc cells determined by FACS analysis, whereby

Figure 2A represents dot blots of heterozygous (+/-) *chico* wing disc cells displaying forward scatter (FSC), which is a measurement for cell size, and side scatter (SSC), which is a measurement for cell granulation, and whereby

Figure 2B is an analogue representation as Figure 2A for homozygous (-/-) *chico* wing disc cells.

Figure 2C is a histogram that displays cell size (FSC) and cell number. Comparison of the mean value of the FSC in Figure 1A and Figure 1B of the gated cell population (R1) reveals that the mean value of the FSC of homozygous mutant *chico* cells is reduced by 10-14 percent in three independent experiments compared with the mean value of the FSC of heterozygous cells.

Figure 3A shows the genomic structure of the *chico* locus at 31B-C with the putative transcriptional start site lying 221 bp 3' from the end of *bsk* encoding DJNK. *ME31B* encodes a DEAD box RNA helicase (de Valoir et al., 1991). Comparison of the genomic and cDNA sequence revealed that the *chico* transcript contains 9 exons with the putative translational start site in the second exon. Black boxes represent translated exons and open boxes indicate untranslated exons. See text for details concerning the P element insertion, the deficiency chromosome, and the rescue constructs. H = HindIII; E = EcoRI.

Figure 3B shows the translated amino acid sequence wherein the black-boxed sequence represents the PH domain and the grey-boxed sequence the PTB domain, respectively, and wherein the putative DRK binding site



(YQN) and the two putative p60 PI3K binding sites (YIPM and YLEM) are highlighted.

Figure 3C shows a PH and PTB domain alignment, wherein the PH domain and the PTB domain of CHICO were aligned to human IRS-1,2,4 and mouse IRS-3, and wherein the dark boxes indicate amino acid identity, while the grey boxes indicate amino acid similarity. The lowercase letters in the consensus line represent identity in 3-4 of the proteins, while upper case letters represent identity in all proteins. The percentage identity was calculated by comparing CHICO individually to each mammalian IRS, and then averaging the four separate identities.

Figure 4A is a tangential section through an eye containing a CHICO (-/-) clone, whereby the *chico* (-/-) clone is recognized by the lack of pigment (bright ? passages). Within the clone, all photoreceptor (PR) cells are reduced in size by about 50 percent compared with wild-type PR cells (dark). At the border of the clone ommatidia composed of wild-type and small *chico*<sup>-/-</sup> mutant PR cells (arrow head) are visible, indicating that CHICO controls cell size autonomously. The numbers represent PR cells R1-R7.

Figure 4B is a comparison of wild-type flies with flies in which the *chico* function in the eye imaginal disc cells has been removed, thereby generating flies with a strongly reduced head capsule and reduced eyes, whereas the proboscis and the rest of the body are of wild-type size. The flies compared were of the following genotypes: (B, left panel) *y w ey-Flp; chico*<sup>1</sup> *FRT40 / P(w<sup>+</sup>) 1(2)2L-3.1 FRT40; P(w<sup>+</sup> *chico* genomic rescue construct pCSR4)/+*, (B, right panel) *y w ey-Flp; chico*<sup>1</sup> *FRT40 / P(w<sup>+</sup>) 1(2)2L-3.1 FRT40*, (C, scanning electron micrograph, left panel) *y w ey-Flp; chico*<sup>1</sup> *FRT40 / CyO*, (C, scanning electron micrograph, right panel) *y w ey-Flp; chico*<sup>1</sup> *FRT40 / P(w<sup>+</sup>) 1(2)2L-3.1 FRT40*.

Figure 4C is an enlarged view of the head region of Figure 4B.

Figure 5A shows the fresh and dry weight of individual wild-type (+/+), heterozygous (+/-), and homozygous (-/-) *chico* mutant males, whereby measurement was made of individual adult males (n = 10) reared under the same growth conditions and analyzed three days after eclosion. The dry weight of wild-type, heterozygous and homozygous mutant males is approximately 28 percent of the corresponding fresh weight.

Figure 5B shows lipid, protein and glycogen contents of the same analytical group as in Figure 1A. As can be seen homozygous *chico* mutant (-/-) males contain almost twice as much lipid calories per milligram fresh weight compared with heterozygous (+/-) or wild-type (+/+) flies.

#### Modes for carrying out the invention

In order to use insects, in particular *Drosophila* mutants as in vivo monitoring system, the function of the defect gene and differences between wild-type *Drosophila* and *chico* mutant *Drosophila* have been studied.

Thereby it was found that *chico* encodes a homologue of vertebrate insulin receptor substrates, IRS1-4.

The insertion site of the P element in *chico*<sup>1</sup> was mapped 1.5 kb downstream of the *bsk* gene (Figure 3A). Isolation and analysis of partial cDNA clones, a full length EST clone and the corresponding genomic sequence flanking the P element insertion, indicated that the *chico* gene consists of a single transcription unit of 3.6 kb with 9 exons. The P element insertion is located 80 bp downstream of the putative translation initiation site in the PH domain (see below). The open reading frame codes for a protein product of 967 amino acids with a

calculated molecular weight of 97 kd. The CHICO amino acid sequence exhibits the strongest similarity with members of a family of vertebrate insulin receptor substrate proteins known as IRS1-4. Vertebrate IRS family members are characterized by an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain and by a number of phosphotyrosine motifs that can serve as docking sites for SH2-containing proteins (for review see Yenush and White, 1997).

10           The cDNA sequence (SEQ ID NOS 1, 2) of wild-type *chico* is shown in Table 1 together with the respective protein sequence, (SEQ ID NOS 2, 3) and the genomic DNA sequence (SEQ ID NO 4) is found in Table 2 below.

15           While the cDNA sequence shows the coding sequence only, the genomic sequence comprises functional flanking parts.

          Furthermore, it has to be understood that all sequence related statements - also without specific  
20 mention - also concerns the complementary strand.

Table 1: cDNA and Protein Sequence

|    |  |         |
|----|--|---------|
|    | 154/1  | 184/11  |
| 5  | 214/21   | 244/31  |
|    | ATG GCA TCA ATA TCG GAT GAC GGC ATG GCG CTG AGT GGC TAC CTC AAG AAG<br>CTG AAG<br>ACC ATG AAG AAG AAG TTC TTT GTG CTG TAC GAG GAG ACG AGC ACT TCG GCA<br>GCC CGG<br>CTG GAG<br>Met ala ser ile ser asp asp gly met ala leu ser gly tyr leu lys lys<br>leu lys<br>thr met lys lys lys phe phe val leu tyr glu glu thr ser thr ser ala<br>ala arg<br>leu glu<br>274/41<br>334/61   |         |
|    |  | 304/51  |
|    |  | 364/71  |
| 20 | TAC TAC GAT ACC GAA AAG AAG TTC CTG CAA AGA GCC GAG CCA AAA AGG GTT<br>ATA TAT<br>CTG AAG AAT TGC TTC AAC ATC AAT CGC CGT TTG GAC ACC AAG CAT AGA TTT<br>GTC ATT<br>GTG CTC<br>tyr tyr asp thr glu lys lys phe leu gln arg ala glu pro lys arg val<br>ile tyr<br>leu lys asn cys phe asn ile asn arg arg leu asp thr lys his arg phe<br>val ile<br>val leu<br>394/81<br>454/101  |         |
|    |  | 424/91  |
|    |  | 484/111 |
| 35 | TCC TCC AGA GAC GGT GGA TTC GGC ATC GTT CTC GAG AAC GAA AAT GAT TTA<br>CGC AAA<br>TGG TTG GAC AAA CTA CTA GTT CTA CAA AGG AAC ATA GCC AAT TCG AAT GGA<br>ACA GCG<br>CAC TCA<br>ser ser arg asp gly gly phe gly ile val leu glu asn glu asn asp leu<br>arg lys<br>trp leu asp lys leu leu val leu gln arg asn ile ala asn ser asn gly<br>thr ala<br>his ser<br>514/121<br>574/141 |         |
|    |  | 544/131 |
|    |  | 604/151 |
| 45 | CCT TAT GAC CAC GTT TGG CAA GTT GTC ATT CAA AAG AAG GGT ATT TCG GAG<br>AAA GTT<br>GGA ATC ACC GGA ACC TAC CAC TGT TGC CTT ACT TCA AAA TCC CTG ACA TTC<br>GTG TGC<br>ATT GGA<br>pro tyr asp his val trp gln val val ile gln lys lys gly ile ser glu<br>lys val<br>gly ile thr gly thr tyr his cys cys leu thr ser lys ser leu thr phe<br>val cys<br>ile gly<br>634/161<br>694/181 |         |
|    |  | 664/171 |
|    |  | 724/191 |

- CCG GAG AAG ACG CCC AAT GGC GAG GAT CGC GTT GCG AGC ATT GAA ATA CTT  
TTG ACC  
ACG ATC AGG CGA TGC GGT CAT GCA TCC CCA CAA TGT ATA TTC TAC GTG GAA  
CTT GGC  
5 CCG CAA  
pro glu lys thr pro asn gly glu asp arg val ala ser ile glu ile leu  
leu thr  
thr ile arg arg cys gly his ala ser pro gln cys ile phe tyr val glu  
leu gly  
10 arg gln 784/211  
754/201 844/231  
814/221
- AGT GTC TTG GGA TCT GGT GAT CTG TGG ATG GAG ACG GAT AAC GCA GCT ATT  
15 GCT ACT  
AAT ATG CAC AAC ACG ATA CTG AGC GCT ATG TCA GCC AAA ACA GAG TCG AAC  
ACG AAT  
TTA ATA  
ser val leu gly ser gly asp leu trp met glu thr asp asn ala ala ile  
20 ala thr  
asn met his asn thr ile leu ser ala met ser ala lys thr glu ser asn  
thr asn  
leu ile 904/251  
874/241 964/271  
25 934/261
- AAC GTT TAT CAG AAT AGA CCT GAC TTA AGT CAC GAG CCC ATG AGA AAG CGA  
TCG TCG  
TCT GCA AAC GAA GCA TCG AAG CCG ATA AAC GTA AAT GTC ATA CAA AAT AGT  
30 CAA AAC  
TCT CTC  
asn val tyr gln asn arg pro asp leu ser his glu pro met arg lys arg  
ser ser  
ser ala asn glu ala ser lys pro ile asn val asn val ile gln asn ser  
35 gln asn  
ser leu 1024/291  
994/281 1084/311  
1054/301
- GAA TTG CGC AGC TGC AGT TCG CCC CAT AAC TAT GGT TTC GGC AGA GAG AGA  
40 TGC GAT  
AGC TTA CCA ACC AGA AAT GGA ACC CTA AGC GAG TCC AGC AAT CAA ACG TAC  
TTT GGT  
TCC AAC  
45 glu leu arg ser cys ser ser pro his asn tyr gly phe gly arg glu arg  
cys asp  
ser leu pro thr arg asn gly thr leu ser glu ser ser asn gln thr tyr  
phe gly  
ser asn  
50 1114/321 1144/331  
1174/341 1204/351
- CAT GGA CTG CGA TCC AAT ACT ATA TCT GGC ATC CGT CCG CAC TCA ACC AAC  
AAG CAT  
55 AGT AAT AGT CCA ACG TTC ACC ATG CCA TTA AGA TGC TCA GAA TCC GAA GAG  
TCA TCA  
ATT AGT  
his gly leu arg ser asn thr ile ser gly ile arg pro his ser thr asn  
lys his

ser asn ser pro thr phe thr met pro leu arg cys ser glu ser glu glu  
 ser ser  
 ile ser  
 1234/361  
 5 1294/381  
 1264/371  
 1324/391  
 GTC GAT GAA TCC GAC GAC AAC GGC AGT TTT AGC CAC TAC AGA TTA AAC ACG  
 CGG TCA  
 TCT GAG ACG GCA ATT CCT GAG GAA AAC ATT GAT GAC TTT GCC AGT GCG GAA  
 10 TTA TTT  
 AGC AAA  
 val asp glu ser asp asp asn gly ser phe ser his tyr arg leu asn thr  
 arg ser  
 ser glu thr ala ile pro glu glu asn ile asp asp phe ala ser ala glu  
 15 leu phe  
 ser lys  
 1354/401  
 1414/421  
 1384/411  
 1444/431  
 20 GTC ACC GAA CAA AAT GTA AGT GAC GAA AAC TAC ATA CCG ATG AAT CCA GTC  
 AAT CCT  
 ACC GAT GCT ATC CAT GAA AAG GAG AAG GCT GAT ATG CAG AGA TTG GAA GAT  
 GCT TCG  
 CTG CAT  
 25 val thr glu gln asn val ser asp glu asn tyr ile pro met asn pro val  
 asn pro  
 thr asp ala ile his glu lys glu lys ala asp met gln arg leu glu asp  
 ala ser  
 leu his  
 30 1474/441  
 1534/461  
 1504/451  
 1564/471  
 TTC AAC TTT CCG GAG CAC GCG TCG GAA AAG CTT GCT AAG GAT TTT GAT CTG  
 GAC TCT  
 35 GAT AAC CAA TGC TGT CGT CCC ATT CGC GCC TAT TCG ATA GGC AAC AAG GTT  
 GAG CAT  
 TTA AAG  
 phe asn phe pro glu his ala ser glu lys leu ala lys asp phe asp leu  
 asp ser  
 40 asp asn gln cys cys arg pro ile arg ala tyr ser ile gly asn lys val  
 glu his  
 leu lys  
 1594/481  
 1654/501  
 1624/491  
 1684/511  
 45 TTT AAT AAG CGC CTG GGA CAC TTG AAT GAT ACG GGA CAG AAT CCG AAT CGC  
 GTG CGA  
 GCC TAC TCG GTT GGC TCC AAA TCG AAG ATA CCG CGC TGC GAC CTG CAG CGA  
 GTG GTC  
 50 CTC GTG  
 phe asn lys arg leu gly his leu asn asp thr gly gln asn pro asn arg  
 val arg  
 ala tyr ser val gly ser lys ser lys ile pro arg cys asp leu gln arg  
 val val  
 55 leu val  
 1714/521  
 1774/541  
 1744/531  
 1804/551  
 GAG GAC AAT AAA CAT GAG TTC ACA GCG AAT AGG AGT CAG AGT AGC ATT ACC  
 60 AAG GAA

GGA ACC AGC TAT GGC AGC AGT GCC AAT CGA CAA AAG AAG TCC ACA AGT GCT  
 CCA CTC  
 CTC AGT  
 glu asp asn lys his glu phe thr ala asn arg ser gln ser ser ile thr  
 5 lys glu  
 gly thr ser tyr gly ser ser ala asn arg gln lys lys ser thr ser ala  
 pro leu  
 leu ser  
 1834/561 1864/571  
 10 1894/581 1924/591

CTG AAG AAC CAG ATA AAC TCC GAC CGA ATG AGT GAC TTA ATG GAA ATT GAT  
 TTT TCA  
 CAA GCA ACC AAT TTG GAA AAG CAG AAG TTC ATC AAG AAT AAT GAA ATT CCG  
 15 AAA TAC  
 ATT GAA  
 leu lys asn gln ile asn ser asp arg met ser asp leu met glu ile asp  
 phe ser  
 gln ala thr asn leu glu lys gln lys phe ile lys asn asn glu ile pro  
 20 lys tyr  
 ile glu  
 1954/601 1984/611  
 2014/621 2044/631

AAC GTG TTC CCA AAA GCC CCG CGA ACG GAT AGC TCC AGC CTA ACT CTG CAC  
 GCC ACA  
 AGT CAA AAG GAC ATT TTC AAT GGC ACC AAA CTA AAT AAC ACT GCG ATC ACA  
 TCC GAG  
 GAT GGT  
 30 asn val phe pro lys ala pro arg thr asp ser ser ser leu thr leu his  
 ala thr  
 ser gln lys asp ile phe asn gly thr lys leu asn asn thr ala ile thr  
 ser glu  
 asp gly  
 35 2074/641 2104/651  
 2134/661 2164/671

TAC CTC GAG ATG AAG CCA GTC GGT AAT GGA TAC ACT CCC AGT TCG AAT TGC  
 CTG CCA  
 40 ATG AAA GTG GAG AAA CTC AAG CTA TCC GAC TAT CAG ACA GCA CCG CCA CTC  
 ACC GCA  
 ACA GCC  
 tyr leu glu met lys pro val gly asn gly tyr thr pro ser ser asn cys  
 leu pro  
 45 met lys val glu lys leu lys leu ser asp tyr gln thr ala pro pro leu  
 thr ala  
 thr ala  
 2194/681 2224/691  
 2254/701 2284/711

GCA CCA GTG CAC GAT TTA AAC AAA ATT AGC ACA TAC AAT ATA TCC GCT GAG  
 AAA TGG  
 AGA GAA CAG CCC AGC AGA AGC GAG GAA AAG AAG TCG AAC TCG CCA TTG AAT  
 GAC AAC  
 55 ACC TTT  
 ala pro val his asp leu asn lys ile ser thr tyr asn ile ser ala glu  
 lys trp  
 arg glu gln pro ser arg ser glu glu lys lys ser asn ser pro leu asn  
 asp asn  
 60 thr phe

2314/721  
2374/741

AGC TCG AAA CCC ACA AAT GTC GAG AGT ACA AGC AAA AGC CAT GAT GTT CAT  
5 TCA GCA  
AAT CAA ATT GAT TGC GAG AAA GTG TGC GCG CAG AGC AGC GAT AAG CTA AAT  
AAT CAT  
CTG GCC  
ser ser lys pro thr asn val glu ser thr ser lys ser his asp val his  
10 ser ala  
asn gln ile asp cys glu lys val cys ala gln ser ser asp lys leu asn  
asn his  
leu ala  
2434/761 2464/771  
15 2494/781 2524/791

GAC AAG ATT GTC GAG AAC AAC AAT TTG GAT ATA GGC GGG CAT GAG GAA AAG  
AAG TTG  
GTT CAT TCG ATA AGC AGC GAA GAC TAC ACA CAA ATC AAG GAC AAA TCG AAT  
20 GAT TTC  
ACA AAA  
asp lys ile val glu asn asn asn leu asp ile gly gly his glu glu lys  
lys leu  
val his ser ile ser ser glu asp tyr thr gln ile lys asp lys ser asn  
25 asp phe  
thr lys  
2554/801 2584/811  
2614/821 2644/831

30 TTT AAC GAA GCC GGC TAC AAA ATT CTG CAA ATT AAA AGC GAC AGC TCA CTC  
ATC TCA  
TCG AAG CTA TAC CAA AAG GGT ATA CAC AAG GAT AAC TTG GAG CGT TCG CAG  
AGA CTT  
ACA GAG  
35 phe asn glu ala gly tyr lys ile leu gln ile lys ser asp ser ser leu  
ile ser  
ser lys leu tyr gln lys gly ile his lys asp asn leu glu arg ser gln  
arg leu  
thr glu  
40 2674/841 2704/851  
2734/861 2764/871

AGT GTG AAT ACG ATT CCC GAT AAT GCC ACC GCC ACC GCG GTG AGC AGC AGC  
TCA CTC  
45 ACC AAA TTC AAT ATA AAT TCA GCA AAG CCA GCC GCC GCC GAT TCG CGT  
AGC ACT  
GGC ACA  
ser val asn thr ile pro asp asn ala thr ala thr ala val ser ser ser  
ser leu  
50 thr lys phe asn ile asn ser ala lys pro ala ala ala asp ser arg  
ser thr  
gly thr  
2794/881 2824/891  
2854/901 2884/911

55 GAT CCA AGT ACA CCA CAG AAC ATT CTA CAG ATT AAA GAT TTG AAT TTC CCC  
TCA AGG  
TCG TCG TCT CGC ATA TCC CAG CCG GAG CTG CAC TAC GCC AGC CTA GAT CTT  
CCC CAT  
60 TGC AGT



asp pro ser thr pro gln asn ile leu gln ile lys asp leu asn phe pro  
ser arg  
ser ser ser arg ile ser gln pro glu leu his tyr ala ser leu asp leu  
pro his  
5 cys ser 2944/931  
2914/921 3004/951  
2974/941

GGC CAA AAT CCA GCT AAA TAC CTG AAG AGA GGA TCA CGC GAA TCG CCG CCG  
10 GTG TCC  
GCA TGC CCG GAG GAT GGG AAT ACC TAT GCG AAA ATC GAC TTT GAC CAA TCC  
GAC TCC  
TCT TCC  
gly gln asn pro ala lys tyr leu lys arg gly ser arg glu ser pro pro  
15 val ser  
ala cys pro glu asp gly asn thr tyr ala lys ile asp phe asp gln ser  
asp ser  
ser ser  
3034/961

20 TCC TCA TCG AAC ATA TTT AAT ACG TAA  
ser ser ser asn ile phe asn thr OCH

Table 2: Genomic DNA sequence

AGAACGACTTTTTCCTCCTTAGTCAGTCACAAGAAAACATAAGCTTACCA  
5 ACAATACGGCGTGTATTGTTAAATTTATTACAACAAAATAAAATATTCAAAT  
TGTATTTAAAAATATAGTAACCATTAATAAAATAAAATCAATATGCGAAAC  
TTTGTAAATTTCTTACTCATCCCTTGTTTTTGAGCCCGCTTCTTAAGTTA  
AATCGTTAAAAATACCAGTTTAAATCATTTCTATTGTCCTGATTTCAGGAGCT  
AATTACATTTTAAATCTTTGTATAAATTCATAAAATTAATAATGGAATGTT  
10 TAACCCACATAAAATATTGGGTATATAAAGTCGATACATATCTTTAAAAAT  
TTTGTTCATACACAAGAATATGGAAGAGTAGATAATTTAGTTACCGATTAAA  
ACATTTCTAAAAATACAAAAATTTAAAAATGATGATTAAAAATAAACTGT  
TATACTAAATTTAAACGAAACAAACGGTCAATTCGATAACTCAATTAGTAT  
CGAATAAGCCGGCGTGTAAATCGGGTTGGCAACTCTCACC GGTTAGAGAG  
15 TCGGGATGGCAACTTCGTATTGTTATTCTTATGCTGCGATAACGATAACA  
CGAAAGTGTCTCGCGAATAAAATGTGGGTTTGATAATAAGAAATTCGGTT  
GGTGAACAATAATAATACAAATGCTAAATGTATCGCGCGGATAAAGTGA  
AACACTGATTTTCGCGCATATCGGGCATACGGGCAGCTAGACGCTTAGGT  
AACACATTTCCCGCCACATTTGGCGTTGAGGTATTATTTCCCATATCCAT  
20 GTGCGTTTGTAAATGATACCCACAGAGTGTGCCATATGTATCGTTGTTTG  
TACATACATGCTACGGGGAAAAATAACTCGCAGATACATATGTATGTAA  
GATGATTATAGAATTCACAGATATATGTACATATAAATATATATATAT  
TTATGATGCTCATCTATAAAACAAAATATGTACAAACATACGGCGCCGACG  
TACTTATGTATGTACATATACATATACATATATGTATAATAAAATGAG  
25 CATCTAGCTGCGGTTATCTTAATGCAATCGCGAGAAACCTGAAAAACGAAA  
TAAACAATCTTTACAGCGCCAGCACAGTGAGCCAAATTATGAATTCACAA  
TCCACATCCAATTCGGATTCCGAAATTCATCGCTTTACATCTTAATTGGA  
ATACGTCGCGCGCGCTAAGCTGCACTCGAATATTGACATAAACACGACGTAA  
TTGCGTGTTTTGATTGCGGATTTCGGATGCTCGATGTTGACAGACGGCAAG  
30 GATTTTTTTGCCAGCCGACATTGCGAATGCTTTTCGCGTGTTCGCTGT  
TGAAAAAGCGATTGTGTTCCGGCAGTTGGAAATGTTTTGTGCTGTTTAT  
GCTTAGAAAAAGCAATGTATTGCACAAAACCTCGTGTTCCTACATTAGTA  
AAGCCTATAACTTAGGTATATGTTTCTAAATTACAATGCAAAAAATAAAA  
AACATTATACATGTGTTGCTTCTTAATTTGAAAAACAGAAAAGTGAAAGC  
35 CTTCGAAATCAAAATATGTGTCCATATCGCCTACTAATAATATAAACACGT  
CGCCTTCCAGGAACCTAAGATTGGAATCATGGCATCAATATCGGATGACG  
GCATGGCGCTGAGTGGCTACCTCAAGAAAGCTGAAGACCATGAAGAAGAA  
TTCTTTGTGCTGACGAGGAGACGAGCACTTCGGCAGCCCGGCTGGAGTA  
CTACGATACCGAAAAAGAGTTCTCTGCAAAAGAGCCAGCCAAAAGGGTTA  
40 TCTATCTGAAGAATTGCTTCAACATCAATCGCCGTTTGGACACCAAGCAT  
AGATTGTGCTATTGTGCTCTCTCCAGAGACGGTGGATTTCGGCATCGTTCT  
CGAGAACGAAAAATGATTTACGCAAAATGGTTGGACAACTACTAGTTCTAC  
AAAGGAACATAGCCAATTCGAATGGAACAGCGCACTACCTTATGGTATG  
CCAAATAAATATAACTACCGATTAGTATGAAACCTAATAACACTTCAAAAT  
45 ACTTTGACAGCCAGGTTTGGCAAGTTGTCTTCAAAAGAAAGGGTATTTCG  
GAGAAAGTTGGAATCACCGGAACTACCACTGTGGCTTACTTCAAAATC  
CCTGACATTCGTGTGCAATTGGACCGGAGAAAGACGCCCAATGGCGGAGGAT  
CGGTTGCGGAGCATGAAATACTTTTGACCAGATCAGGCGGTTAGTTGTT  
GCCAGCAAACTGCAAGGGATTGTAAATAATTCGGACTTAATTCAGAT  
50 GCGGTCATGCATCCCCACAAATGTATATTTCTAGTGAAGTAAATTCAGAT  
AGTGTCTGGGATCTGGGTATCTGTGGATGGAGACGGATAACGCAGCTAT  
TGCTACTAATATGCAACAACGATAGTGAAGTATTAGCTCTCATTACAA  
CTAATCCAAAGATTTCATGATCATCTCAAAACGACATAGATAGTTAAAG  
ATATCTCCAGTTAACTTTAATAATTTCTGTGGGTTTTTCTTTCAAGCGCT  
55 ATGTGACCCAAAACAGAGTCGAACACGAATTTAATAAACGTTTATTCAGAA  
CAGACCTGACTTAAGTCACGAGCCATGAGAAAGCGATCGTCGTCTGCAAC  
ACGAAGCATCGAAGCCGATAAACGTAATGTCATACAAAATGATCAAAAC

TCTCTCGAATTGCGCAGCTGCAGTTCGCCCCATAACTATGGTAAATCTT  
CAAAATGTATGTTTAAACGCAAAATTAATCAAAACGCAATCGTTTCAGGTTT  
CGGCAGAGAGAGATGCGATAGCTTACCAACCGAGAAATGGAACCCCTAAGCG  
AGTCCAGCAATCAAAACGTACTTTGGTTTCCAACCTGGACTGCGATCCAATT  
5 ACTATATCTCGCATCCGTCGCCACTCAACCAACAAGCATAGTAATATGTC  
AACGTTTACCAATGCCATTAAGATGCTCAGATCCGAAGATCTCAATTAA  
GTGTGATGAATCCGACGACAACGGCAGTTTTAGCCACTACAGATTAAAG  
TGCGTTGCTATCAAATAATAATTATTTAATAATAATCACCATTTCATTT  
CTAGCAGCGGTCATCTGAGACGGCAATTCCTGAGGAAAAACATTGATGAC  
10 TTTGCCAGTGCGGAATTATTTAGCAAAGTCAACCGAACAAAATGGTAAGCG  
AAACACAAAAACAATTTTTTAACATGAAAAGTAGCTAATCAATTGGCTTT  
GTTTAACTGCAGTAAGTGACGAAAACTACATACCGATGAATCCAGTCAAT  
CCTACCGATGCTATCCATGAAAAGGAGAAGGCTGATATGCAGAGATTGGA  
AGATGCTTCGCTGCATTTCAACTTTCCGGAGCAGCGCTCGGAAAAAGCTTG  
15 CTAAGGATTTTGTCTGGACTCTGATAACCAAGTGAGTACACATTTCCGTT  
CAACTTGGCACGTAATGCAATCAATCAATCTTTGTTACAGATGCTGTGC  
TGCCATTTCGCGCTATTCGATAGGCAACAAGTTGAGCATTTAAAGTTTA  
ATAAGCGCTGGGACACTTGAATGATACGGGACAGAAATCCGAATCGCGTG  
CGAGCCTACTCGTTGGCTCCAATCGAAGATACCGCGCTGGGACCTGCA  
20 GCGAGTGGTCTCGTGGAGGACAATAACATGAGTTACAGCGGAATAGGA  
GTCAGAGTAGCATTTACCAAGGAAGGAACCGCTATGGCAGCAGTGCCAAAT  
CGACAAAAGAAAGTCCACAAGTGCTCCACTCCTCAGTCTGAAGAACCGAT  
AACTCCGACCGAATGAGTGACTTAATGGAAATTGATTTTTCAACAGCAA  
CCAATTTGAAAAGCAGAAAGTTTCATCAAGAAATAATGAAATTCGAAATAC  
25 TCTGCAGCCCAAGTCAAAAGGACATTTTCAATGGCACCAAACTAAATA  
ACACTGCGATCACATCCGAGGATGGTTACTCGAGATGAAGCCAGTGGGT  
AATGGATACACTCCAGTTTCGAATTGCTTGCCAATGAAAGTGGAGAGGCT  
CAAGCTATCCGACTATCAGACAGCACCCGCAATCACCGCAACAGCCGCAC  
30 CAGTGCACGATTTAAACAAAATTAGCACATACAATATATCCGCTGAGAAA  
TGAGAGAAACAGCCAGCAGAGCGAGGAAAAGAAAGTCCGAATCGCCATT  
GAATGACAACACCTTTGGCTTGAAACCCACAATATGTCGAGAGTACAAGCA  
AAAGCCATGATGTTCAATTCAGCAAAATCAAATTTGATTCGAGAAAAGTGTGC  
GCGCAGAGCAGCGATAAGCTAAATAATCTGGCCGACAAGATTTGTCGAGAA  
35 CAACAATTTGGATATAGGCGGGCATGAGGAAAAGAAAGTTGTTTCAATCGA  
TAAGCAGCGAAGACTACACACAATCAAGGACAAATCGAATGATTTACA  
AAATTTAACGAAGCCGGCTACAAAATTTCTGCAATTTAAAGCGACAGCTC  
ACTCATCTCATCGAAGCTATACCAAAAGGGTATACACAAGGATAACTTGG  
AGCGTTCGAGAGACTTACGGAGAGTGTGAATACGATTCCCGATAATGGC  
40 AGCCGACCGCGGTGAGCAGCAGCTCACTACCAAAATTCATATATAATTC  
AGCAAAAGCCAGCCGCGCGCCGCGGATTGCGGTAGCACTGGCACAGATCCAA  
GTACACACAGAAACATTTCTACAGATTAAAGATTGAAATTTCCCTCAAGG  
TCGTGCTTCGCATATCCAGCCGGAGCTGCACTACGCCAGCCTAGATCT  
TGCCCAATTGCAAGTGCCAAAAATCCAGCTAAATACCTGAAGAGAGGATCAC  
45 AAAATCGACTTTGACCAATCCGACTCTCTTCTCTCATCGAACATATTG  
TAATACGATAAAGTTTGAATTTATGACCTATCCTATATATATGATTTG  
TTTAATATTGTACATTTATTGTAATATTCTCTGACAAGCAAGCTTACA  
ATTTTGGATGCTAATAAAATAAATTTTATTAATTTATAATGATCCCTTTG  
50 GACTTTTTTTTTTTTGGACTAAGAAATCACTACTAAAGAAGGGCTTTT  
GAGGGTTAAA

Sequence similarity between CHICO and IRS1-4 is confined to the N-terminal region including the PH domain and the PTB domain. The amino acid identity is 41 percent in the PH domain and 38 percent in the PTB domain (Figure 3B and C). Although there is no significant overall homology within the C-terminal domain, the CHICO protein contains several putative SH2 binding motifs characteristic for IRS family members. Two motifs at positions 411 and 641 fit the consensus binding site (YXXM) for the p85/p60 adaptor subunit of PI(3)K (Songyang et al., 1993; Weinkove et al., 1997) and one (at position 243) corresponds to the consensus (YXN) for GRB2/DRK binding (Olivier et al., 1993; Songyang et al., 1993).

Owing to the insertion of the P element 80 bp downstream of the translation start site *chico*<sup>1</sup> is likely to be a null mutation. The *chico*<sup>2</sup> allele is a synthetic deletion allele covering the putative translation start site and the regulatory region of *chico* (Figure 3A; see experimental procedures). Flies homozygous for this synthetic *chico* null allele were viable and showed phenotypes indistinguishable from flies homozygous for *chico*<sup>1</sup> (Figure 1B). The mutant phenotype of both alleles is fully rescued by an 8 kb genomic rescue construct encompassing the *chico* transcription unit and its endogenous regulatory sequences (Figure 3A). Therefore the reduction in body size and the female sterility is caused by the loss of *chico* function.

In order to profit from the close similarity of *chico* with IRS 1-4, clearly detectable, and preferably quantifiably differences between *chico* mutants and wild-type flies must exist.

Therefore, specific differences have been searched for and studied.

To quantify size differences in various mutants, the weight of individual flies was determined (Figure 1B). Flies homozygous for the P element (*chico*<sup>1</sup>)

or a synthetic *chico* deletion (*chico*<sup>2</sup>) have a drastic weight reduction (by 65% in females and 55% in males) compared with wild-type control flies of the same age. Body size reduction is observed at all developmental stages, but does not alter the overall proportions of the flies (Figure 1A).

Since the reduction in body and organ size in *chico* mutants could be due to a reduction in the number of cells and/or a reduction in the size of the individual cells, cell number and cell size in the wing was determined. In the wing, each epithelial cell secretes cuticle with a single hair, so that counting the number of hairs and determining their density provides a direct measure of cell number and cell size in the wing. (See Table 3)

**Table 3:** Cell Size and Cell Number are Affected in Wings of Homozygous Mutan Animals<sup>a</sup>

| Genotype                                   | 1<br>Area <sup>b,c</sup><br>(10 <sup>6</sup> µm <sup>2</sup> ) | 2<br>Overall<br>size<br>reduction<br>(%) | 3<br>Cell density <sup>d</sup><br>(cells/µm <sup>2</sup> ) | 4<br>Area<br>covered<br>per<br>cell <sup>e</sup><br>(µm <sup>2</sup> ) | 5<br>Cell size<br>reduction<br>(%) | 6<br>Approx. No. of<br>cells in measu-<br>red area <sup>b,f</sup> | 7<br>Cell<br>number<br>reduction<br>(%) |
|--|--|--|--|--|------------------------------------|---|---|
| Y,w; +/Sp                                  | 1.47±0.009   | -----                                    | 6.38x10 <sup>-3</sup> ±0.1x10 <sup>-3</sup>                | 157±2.36   | -----                              | 9379  | -----                                   |
| Y,w;chico <sup>2</sup> /Sp                 | 1.40±0.018   | 4.8                                      | 6.32x10 <sup>-3</sup> ±0.1x10 <sup>-3</sup>                | 158±2.90   | 0                                  | 8848  | 5.7                                     |
| Y,w;chico <sup>2</sup> /chico <sup>2</sup> | 0.89±0.009   | 39.5                                     | 7.70x10 <sup>-3</sup> ±0.1x10 <sup>-3</sup>                | 130±1.66   | 17.2                               | 6853  | 27                                      |

<sup>a</sup> From females at least eight wings of each genotype were analyzed.

<sup>b</sup> The area of the whole wing was integrated exclusive the alula and the costal cell.

<sup>c</sup> Measured using NIH Image 1.60.

<sup>d</sup> Assessed by counting number of wing hairs on the dorsal wing surface in a 10 000 µm<sup>2</sup> area just posterior to the PCV.

<sup>e</sup> Reciprocal of column 3.

<sup>f</sup> Generated by multiplying the values in column 1 by those in column 3.

As shown in Table 3, the 40 percent reduction in the size of *chico* mutant wings is caused by a reduction in both cell number and cell size. Reduction in cell number accounts for 68 percent of the total reduction in wing size. The remaining 32 percent of the reduction in wing size is due to a reduction in the average size of mutant cells. Similar results were obtained for the eye. It could be shown that in homozygous mutant *chico* flies, ommatidial number is reduced by approximately 40 percent: homozygous *chico* flies have only about 480 ommatidia per eye (Table 4) whereas wild-type flies have approximately 780 ommatidia per eye. Therefore, loss of *chico* function reduces body size by means of reducing cell number and cell size.

To test whether the reduction in the size of *chico* mutant cells is also observed during larval stages third instar wing discs of larvae homozygous or heterozygous for *chico* were dissociated and the relative cell size of the two cell populations was determined by FACS analysis (Figures 2A to 2C). A 10-14 percent reduction in the mean of the forward scatter of homozygous *chico* cells compared with heterozygous cells indicates that the size of *chico* imaginal disc cells is also reduced.

Since the effect of loss of *chico* function on the overall body and organ size could be due to a non-autonomous role of *chico* in humoral growth regulation or to an autonomous role in a tissue and cell type specific manner, the autonomy of *chico* was investigated. To test the cell autonomy of the *chico* mutation, clones of genetically marked homozygous mutant *chico* cells in a heterozygous background in the eye were generated (Figure 4A) using the *hs-FLP/FRT* system (Xu and Rubin, 1993). In each ommatidium, the R1-R6 photoreceptor cells are arranged in an asymmetric trapezoid. The tall side of the trapezoid is formed by photoreceptors R1-R3 facing

anteriorly. The centrally located R7 photoreceptor has a smaller rhabdomere than the six outer cells. Each of these morphological characteristics is retained in *chico* mutant ommatidia. Thus, loss of *chico* function does not  
5 impair the specification of cell fate. However, it is striking that the size of each mutant photoreceptor and hence the entire ommatidial unit in the mutant clone, is reduced by more than 50 percent. On the periphery of the clones of homozygous mutant tissue, ommatidia consist of  
10 homozygous and heterozygous cells. The genotype of each photoreceptor can be assessed by the presence or absence of red pigment. Small homozygous mutant photoreceptor cells (arrowheads in Figure 4A) coexist with heterozygous cells in the same ommatidium. Remarkably, this does not  
15 significantly alter the shape of the ommatidia and the arrangement of the photoreceptors. Autonomy of cell size control is also observed in the wing (data not shown). Therefore, final cell size is autonomously dependent on *chico* function in each individual cell.

20 In order to further study the effects of *chico*, it was tested whether *chico* affects the size of organs and body parts autonomously. For this testing, *chico* function was selectively removed in the eye imaginal disc using the *eye-FLP* technique (see Example  
25 4). The results presented here, show that selected removal of *chico* function in the head using the *eye-FLP* technique reduces head size. This reduced head size phenotype can be used to identify mutations in other genes that affect growth and thus are likely to encode  
30 components of the insulin signalling pathway. These mutations thus identify genes that are potential drug targets for human diseases such as type-2 diabetes. The eye imaginal disc gives rise to the compound eye and the head capsule but not to the proboscis. In embryos  
35 heterozygous for *chico*, mitotic recombination was selectively induced in the eye progenitor cells by using an FLP recombinase driven by the *eyeless*-enhancer



(Quiring et al., 1994). Owing to the presence of a recessive mutation affecting cell survival on the *chico* chromosome, *chico* homozygous mutant cells have a proliferative advantage and contribute to the majority of cells in the eye and the head. Thus flies have heads that are largely homozygous for *chico* while the rest of the body is heterozygous. In such flies, the eyes and the head capsule are strongly reduced in size while the proboscis and the rest of the body are of wild-type size (Figure 4B, C). Thus *chico* acts autonomously in the control of cell size and organ size.

It was furthermore investigated whether the reduction in cell number caused by the absence of *chico* function is the result of a prolonged cell cycle time or of an increased rate of apoptosis during development. In order to analyze the behavior of *chico* mutant cells during development, genetically marked homozygous mutant cells were generated by mitotic recombination. This allowed comparison between the behavior of homozygous mutant clones and their wild-type sister clones, called twin spots, generated during mitotic recombination. Three differences between mutant and wild-type twin clones are obvious: first, *chico* mutant clones are rare: in approximately 90 percent of the clones, only the darkly pigmented wild-type twin spot can be detected. This is most likely due to the fact that small mutant clones encompass only a few ommatidia and escape detection. Secondly, when a non-pigmented mutant clone is detected, the clone is variable in size and often significantly smaller than the wild-type sister clone. Thirdly, there are regional differences in the frequency of mutant clones: clones are more frequently observed in the anterior half of the eye around the equator. The equator defines a line of dorso-ventral mirror image symmetry in the orientation of the ommatidial units. It appears that mutant cells have a better chance to grow or survive in the center of the eye than on its periphery. The behavior

of *chico* mutant clones is similar to that of *M* mutant clones and has been described as cell competition (Morata and Ripoll, 1975; Simpson, 1979). It indicates that the development of *chico* mutant cells is selectively impaired compared with wild-type cells and that there are regional differences in the ability of mutant cells to grow or survive.

In addition, mutant clones in the eye and wing imaginal discs were tested to investigate whether the reduced size of *chico* mutant clones observed in the adult is due to a growth disadvantage or to impaired cell survival during the final stages of differentiation. This investigation was performed on eye imaginal discs containing *chico* mutant or wild-type control clones, marked by the absence of the green *arm-lacZ* staining, and their intensely bright green staining twin spots. As seen in the adult, the mutant clones are smaller than their twin spots and are variable in size. The clones often form a thin line. The fact that *chico* mutant clones in the third instar disc and in the adult eye exhibit a similar behavior argues against the possibility that homozygous mutant *chico* cells are eliminated during differentiation in the pupal stage.

As a critical determinant of organ size through counterbalancing cell proliferation, apoptosis has been postulated (Conlon and Raff, 1999). In order to test whether programmed cell death contributes to size control by reducing cell number, discs containing either *chico* mutant clones or wild-type control clones were analyzed by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labelling (TUNEL). No significant difference in occurrence of apoptotic cells between wild-type and mutant clones was detected (data not shown). Since *chico* mutant clones are rather small, mutant clones in a *Minute* background were induced. Even though such clones were greatly enlarged due to their growth advantage they also did not reveal enhanced

apoptosis compared with wild-type control clones in a Minute background (data not shown). Furthermore, no increase in morphological signs of programmed cell death such as enlarged cells or cells with picnotic nuclei in *chico* mutant clones was observed, neither in the imaginal discs nor in the adult eye. These results are also consistent with the FACS analysis of heterozygous and homozygous *chico* mutant wing disc cells. No significant difference in the apoptotic sub-G1 fraction of homozygous *chico* mutant cells compared with heterozygous cells was detected (data not shown). Therefore, these results show that *chico* function is not necessary for cell survival, but is required for cell growth and cell proliferation throughout development. Homozygous *chico* mutant cells have a selective growth disadvantage: they grow more slowly than wild-type cells, as indicated by their under-representation in discs and in the adult eye, and they cannot reach the normal size of wild-type cells. The cell cycle profiles of heterozygous and homozygous *chico* mutant wing disc cells, however, are similar (data not shown) suggesting that the increased cell cycle time of *chico* mutant cells is caused by proportional expansion of the G1, S and G2 phase of the cell cycle.

It was also found that *chico* interacts with other essential compounds. Such compounds with which *chico* genetically interacts are the Drosophila insulin receptor and PI(3)kinase. Due to the found homology of CHICO with mammalian IRS1-4, genetic interactions with other components involved in signaling via IRS proteins, such as the insulin receptor and the p110 PI3kinase (PI3K), can be studied. Loss of function mutations in *Inr* are lethal but certain heteroallelic combinations survive to adulthood. Such *Inr* mutant flies are reduced in size (Chen et al., 1996). It was found that, like in *chico* mutants, cell size is reduced by 28 percent in *Inr<sup>313</sup>/Inr<sup>327</sup>* flies. Furthermore targeted expression of a dominant negative variant of Drosophila p110 PI3K in the

developing eye or wing causes a reduction in cell size in the eye, and in both cell size and cell number in the wing. Conversely, overexpression of a constitutively active, membrane-targetted version of PI3K increases cell  
5 size and cell number (Leevers et al., 1996). In flies that are homozygous for *chico*, heterozygosity for a hypomorphic *Inr* allele led to a further reduction in cell number in the wing and the eye (see Table 4).

10

15

**Table 4:** One Mutant Copy of the *Inr* Enhances the Growth Phenotype of *chico* Mutant Cells

| Wing Analysis <sup>a</sup>                                    |   |                                     |   |  |                               |   |                                 |
|---|---|-------------------------------------|---|--|-------------------------------|---|---------------------------------|
|   | 1   | 2                                   | 3   | 4  | 5                             | 6   | 7                               |
| Genotype  | Area <sup>b,c</sup><br>(10 <sup>6</sup> μm <sup>2</sup> ) | Overall<br>size<br>reduction<br>(%) | Cell density <sup>d</sup><br>(cells/μm <sup>2</sup> )                 | Area<br>covered<br>per cell <sup>e</sup><br>(μm <sup>2</sup> ) | Cell size<br>reduction<br>(%) | Approx. No.<br>of cells in<br>measured<br>area <sup>b,f</sup> | Cell number<br>reduction<br>(%) |
| <i>chico</i> <sup>2</sup> ;+/+                                | 0.96±0.03   | -----                               | 8.0x10 <sup>-3</sup> ±0.12x10 <sup>-3</sup>                           | 125±1.9  | -----                         | 7680  | -----                           |
| <i>chico</i> <sup>2</sup> ;<br><i>Inr</i> <sup>05545</sup> /+ | 0.79±0.03   | 17.7                                | 7.8x10 <sup>-3</sup> ±0.09x10 <sup>-3</sup>                           | 128±1.5  | 0                             | 6162  | 19.8                            |
| Eye Analysis <sup>g</sup>                                     |   |                                     |   |  |                               |   |                                 |
| Genotype  | 1<br>Eye area <sup>c</sup>                                | 2<br>No. of<br>ommatidia per<br>eye | 3<br>Area covered per<br>ommatidium <sup>h</sup><br>(arbitrary units) |  |                               |   |                                 |
| <i>chico</i> <sup>2</sup> ;+/+                                | 261 896±5097  | 483±7                               | 542   |  |                               |   |                                 |
| <i>chico</i> <sup>2</sup> ;<br><i>Inr</i> <sup>05545</sup> /+ | 198 634±3280  | 411±2                               | 483   |  |                               |   |                                 |

<sup>a</sup> From females four wings of each genotype were analyzed.<sup>b</sup> The area of the whole wing was integrated exclusive the alula and the costal cell.<sup>c</sup> Measured using NIH Image 1.60.<sup>d</sup> Assessed by counting number of wing hairs on the dorsal wing surface in a 10 000 μm<sup>2</sup> area just anterior to the PCV.<sup>e</sup> Reciprocal of column 3.<sup>f</sup> Generated by multiplying the values in column 1 by those in column 3.<sup>g</sup> Eight eyes of each genotype were analyzed.<sup>h</sup> Generated by dividing the values in column 1 by those in column 2.

Thus, in the absence of *chico* function a reduction of the receptor level potentiates the growth reduction. This CHICO independent signaling of INR is likely to be mediated by PI3K binding sites in the C-terminal tail of the INR (Yenush et al., 1996, see discussion). Similarly, expressing a catalytically inactive version of PI3K in *chico* homozygous wing discs leads to a further reduction in wing size by 48 percent (data not shown). Thus the *chico* mutant phenotype is modified by mutations in *Inr* and PI3K. This is consistent with the notion that INR, CHICO and PI3K form a conserved signaling pathway involved in the cell autonomous control of growth and cell size in *Drosophila*.

Besides of its influence on cell size and cell number, it was found that CHICO also controls lipid levels. This finding is further evidence on the relevance of *chico* in the insulin signalling pathway, since said pathway is known for its role in the control of cellular metabolism in vertebrates and in *C. elegans*. Thus it was investigated whether energy stores are altered in *chico* mutant flies. The fresh and dry weights of different flies were determined (Figure 5A) as well as their amounts of lipid, protein and glycogen per unit of fresh weight (Figure 5B). While there was no significant difference in levels of proteins and glycogen, lipid levels were increased significantly in *chico* males (Figure 5B). In fact, in spite of their smaller size, *chico* males had almost twice as much lipids as wild-type males per mg fresh weight. The dramatic increase in lipids in *chico* mutant males is reminiscent of hypertriglyceridemia in IRS-1 deficient mice (Abe et al., 1998) and of fat accumulation observed in *C. elegans* containing a mutation in the *daf-2* gene, which encodes the insulin receptor homologue (Kimura et al., 1997). Thus it appears that the INR signaling pathway controls cellular metabolism in vertebrates, nematodes and insects.

Since it could be shown that loss of function mutations in *chico*, which encodes a *Drosophila* homolog of IRS 1-4, cause a reduction in overall growth by reducing cell size and cell number, since it could furthermore be shown that the primary function of CHICO is to regulate overall growth by coordinating the control of cell cycle progression and cell growth and not by controlling apoptosis, and since CHICO furthermore is likely to be part of a nutritional sensing system, *chico* and its mutations are very useful tools for the investigation of e.g. the insulin signalling pathway, the screening of potential drugs for the treatment of defects in said pathway, and the screening of drug targets. *Chico* and its mutations are especially suitable tools in *Drosophila* mutants usable as in vitro monitory systems.

Thus, one subject of the present invention is a method for searching for compounds or mutations interacting directly or indirectly with the insulin signaling pathway, characterized in that a viable insect is treated with at least one compound or with at least one mutation generating means, and that the effect of such treatment on the body size and/or cell size and/or development time and/or lipid level is determined whereby alterations of the body size and/or cell size and/or development time and/or lipid level are detectable in at least part of the animal. In a preferred embodiment said method is a method for searching for compounds or mutations interacting directly or indirectly with CHICO involving life processes, whereby a viable *chico* mutant insect, e.g. a fly such as *Drosophila*, is treated with at least one compound or with at least one compound generating means, and that the effect of such treatment is detected, whereby said mutant comprises at most one wild-type *chico* gene. As already mentioned above, the animals suitable in the scope of the present invention are insects. The advantage of insects is the highly conserved insulin signaling pathway together with their

fast reproducibility. Preferably said method is performed with a *Drosophila* mutant, whereby said mutant is treated in the egg or larvae stadium with said compound or compound generating means. As was shown above, different  
5 easily detectable and also quite well quantifiable characteristics can be used to determine the effect of an applied treatment, such as alteration in size and/or weight and/or developmental time and/or lipid levels. Of course, for many applications it is desirable that the  
10 animals used are already altered in size and/or weight and/or developmental time and/or lipid levels, so that therapeutical effects can be detected.

Mutants suitable for the method of the present invention are such ones that do not comprise a  
15 wild-type *chico* gene or that have one wild type *chico* gene. Thus the following combinations are encompassed by the present invention:

- both *chico* genes are totally lost,
- both wild-type *chico* genes are replaced by  
20 *chico* alleles with reduced CHICO activity
- both wild-type *chico* genes are replaced by *chico* alleles with no CHICO activity,
  - one *chico* gene is totally lacking and one is replaced with a *chico* allele having no CHICO activity,
  - 25 - one *chico* gene is totally lacking and one is replaced with a *chico* allele having reduced CHICO activity,
  - one *chico* allele has no activity and one *chico* allele has reduced activity,
  - 30 - one *chico* is the wild-type *chico* gene, and the second *chico* is an allele with no activity
  - one *chico* is the wild-type *chico* gene, and the second *chico* is an allele with reduced CHICO activity
  - one *chico* is the wild-type *chico* gene, and  
35 the second *chico* is totally lacking.

As wild-type CHICO in the scope of the present invention any protein is meant that has the same



effect as wild-type CHICO and at least about 50 % identity with the protein structure of Table 1. As wild-type *chico* any *chico* gene is referred to that encodes the amino acid sequence of Table 1 (2, 3), or that encode a protein which is sufficiently homologous to CHICO that it has the same effect in the animals of the present invention as CHICO. Relevant effects of CHICO - as it results from the above disclosure - are cell size, cell number and lipid level. Preferred DNA sequences are e.g. the genomic or the cDNA sequence represented in Table 2 (SEQ. ID. NO. 4) or Table 1 (SEQ. ID. No. 1, 2).

As *chico* mutation according to the present invention any mutation is considered that has an at least reduced activity compared to the activity of the wild type animal. Preferred *chico* mutations are those having no activity and leading to size reduced homozygous animals. Such *chico* mutations are the sequences resulting in a cell number reduction of at least 10 %, preferably at least 30 %, a cell size reduction of at least about 10 %, preferably at least about 30 %, and an enhancement of the lipid content per weight unit of at least about 20 %, preferably about 50 % (all % concerning homozygous *chico* mutant animals in comparison with wild-type animals). Preferred examples for such a mutation are the mutations described as *chico*<sup>1</sup> and *chico*<sup>2</sup>.

In general, total loss of *chico* activity is obtained when e.g. at least the PH domain is deleted or tyrosin residues Tyr(411) and Tyr(641) (positions referred to SEQ ID NOS 2 and 3) are substituted. Partial loss of CHICO activity is e.g. due to substitutions in the region of the PTB domain. The above mentioned alterations in *chico* are by no means complete and have to be clearly understood as non limiting examples.

The animals of the present invention can e.g. be obtained by mutagenesis procedures known to the expert. Suitable mutagens include but are not limited to P-elements, X-ray and ethylmethane sulfonate (EMS).

A suitable method for generating mutant insects comprises that adult insects, in particular males, are treated with a mutation generating means under mutation generating conditions, that thus treated insects  
5 are crossed to wild-type or mutant insects, in particular to *chico* mutant insects, and that viable offsprings with altered cell number and /or cell size and/or developmental time and/or lipid levels are cultivated under suitable conditions.

10 As it has been shown in the scope of the present invention, it is not necessary to generate fully mutant animals. By e.g. the eye-FLP technique, it is possible to selectively generate mutated head regions. Such partially mutated animals are advantageous for many  
15 applications, since they avoid the laborous need to generate mutated strains.

Specific and much preferred applications of the animals and the method of the present invention are the screening for compounds that are useful in the  
20 modulation, e.g. the treatment, of diabetes type 2, and the further investigation of diabetes type 2, in particular the search for further factors involved in the development of said disease. Such factors possibly involved in the development of said (but also other)  
25 disease can be found by applying a mutation generating means, in particular a mutagen such as radiation, P element, or chemical compound, to the animal under conditions suitable to generate gene defects in the factors

30 One very interesting application of *chico* mutant insects or insects with *chico* phenotype is connected with the fact that the insulin signaling pathway is conserved in structure and function. In mammalian cells, activation of the insulin or IGF-1  
35 (insulin-like-growth factor-1) receptor by insulin and IGF-1, respectively, results in the recruitment of IRS-1 or IRS-2 to the receptor via interaction of the IRS PTB

domains with a phosphotyrosine motif (NPXY) in the juxtamembrane region of the receptors. Phosphorylation of multiple tyrosine residues of IRS-1 triggers the activation of various signaling pathways including the RAS/MAP kinase pathway via the SH2/SH3 adaptor GRB2, and the PI3K/PKB pathway via the p85 SH2 adaptor subunit of p110 PI3K (Yenush and White, 1997). The *Drosophila* INR shares many structural features with its human homologues, including its heterotetrameric structure and a conserved PTB consensus binding site in the juxtamembrane region. Although, the *Drosophila* INR contains a 400 amino acid C-terminal extension not found in any of the vertebrate receptors, it is nevertheless an important tool not only for screening compounds activating the insulin signalling pathway, but also for finding and/or further investigating compounds that are also members of said pathway. However, also the C terminal extension is of importance. This C-terminal tail contains three YXXM consensus binding sites for the SH2 domain of the p60 subunit of PI3K and four additional NPXY consensus PTB binding sites. The C-terminal domain is functional, since expression of a chimeric receptor consisting of the extracellular domain of the human INR and the intracellular domain of the *Drosophila* INR in murine 32D cells lacking endogenous IRS-1 can partially activate mammalian PI3K and S6K. In contrast, the ability of the human INR to activate PI3K in this system is strictly dependent on the coexpression of IRS-1 (Yenush et al., 1996). These findings and the identification of CHICO suggest that in e.g. *Drosophila* INR couples to the downstream effector PI3K in two different ways, one using docking sites in the INR C-terminal tail and the other connecting through docking sites in CHICO.

As described here, the effects on growth and cell size of *chico* mutants are remarkably similar to the phenotypes of mutations in genes encoding other components of the INR pathway in e.g. *Drosophila*.

Although loss of function mutations in the *Drosophila INR* gene are lethal, certain heteroallelic combinations are viable and show delayed development, reduced body size and decreased cell number (Chen et al., 1996) and cell size (this application). Expression of dominant negative or constitutively active variants of p110 PI3K in the developing wing and eye reduces or increases cell number and cell size, respectively (Leevers et al., 1996). Furthermore viable mutations in the gene encoding *Drosophila Protein Kinase B* (Staveley et al., 1998) cause a reduction in cell number and cell size (H.S. and E.H., unpublished results). The striking similarities between the phenotypes of *chico* and mutations in the genes encoding *INR* and *DPKB*, as well as the genetic interactions between mutations in *Inr*, *chico* and *PI3K*, show the specific role of the *INR* pathway in control of cell growth and cell number as a process independent of pattern formation and makes not only flies with a *chico* mutation induced phenotype a valuable in vivo monitoring system, but also flies with a *chico* mutation and at least one further mutation as described above.

Thus, the *chico* mutant animals of the present invention are an especially useful tool to investigate the insulin signaling pathway and possible pharmaceuticals to overcome defects therein, since said pathway is highly conserved from vertebrates to *Drosophila*, not only in regard to its structure but also to its function.

30

### Examples

#### Example 1: *Drosophila* strains

*chico*<sup>1</sup> is a P element insertion allele, originally called *fs(2)4*<sup>1</sup> (Berg and Spradling, 1991). The P element was mapped using standard PCR with primers specific to the 3' end of the P element and to the

genomic sequence. Subsequently, the insertion site was precisely determined by sequencing the amplified PCR fragment. *chico*<sup>2</sup> was derived from *chico*<sup>1</sup> by mobilizing the P element. The resulting *Df(2L)flp147E* deletes the translation start site and the regulatory region of *chico* and the 3' coding sequences of *bsk*. The *bsk* mutation was complemented by insertion of a *bsk* rescue construct on the *Df(2L)flp147E* chromosome (Riesgo et al. 1996). For genetic interaction analysis we used *Dp110*<sup>D954A</sup>, a dominant negative form of p110 of *Drosophila* PI3K (Leevers et al., 1996) and *Inr*<sup>D5545</sup>, a P element induced hypomorphic allele (Fernandez et al., 1995). The *Dp110*<sup>D954A</sup> transgene was driven by GAL4 which was expressed in the dorsal wing pouch using the MS-1096 line (Capdevila and Guerrero, 1994). The *Inr* allele is hypomorphic and recessive lethal.

#### Example 2: Molecular characterization of

##### *chico*

An 11 kb genomic DNA fragment which has been described in Riesgo-Escovar et al., 1996 and encompasses the Jun kinase (*bsk*) and *chico* transcription units was used to screen a *Drosophila* cDNA library. From this screen, a partial cDNA (U1) for *chico* was recovered, sequenced, and used to screen an embryonic *Drosophila* cDNA library. Several cDNAs were isolated and partially sequenced (U2-U4). Sequence search of the *Drosophila* EST database with these sequences identified the EST GH02661. Sequencing of this EST clone indicated that it represents a full-length *chico* transcript that contains a consensus sequence for translation initiation (Cavener, 1987) and ends with a poly A tail 15 bp after a consensus poly-A addition signal. All cDNAs were found to encode the same transcript. The 11 kb genomic region was fully sequenced to establish the exon/intron structure of *chico*, and also its position in relation to *bsk* and *ME31B*. From a genomic phage of the region, a BamHI/ BamHI fragment was

- subcloned into pBluescript, and a resection from the *bsk* side was performed to generate a 9.5 kb fragment that was subcloned into a transformation vector and used to generate a genomic rescue construct for *chico*. pWAX (described in Riesgo-Escovar et al., 1996) rescued both the phenotypes of *chico* and *bsk* separately and in a double mutant (data not shown, and Riesgo-Escovar et al., 1996).

#### Example 3: Weight Analysis

- Body weight of individual male and female flies ( $n = 20$ ) was measured with a precision scale (range 0.001 - 10 mg; Mettler ME30). Flies were reared under the same growth conditions and were age-matched (two days old) before weighing. The genotypes analyzed were the following:  $y\ w; +/+$ ,  $y\ w; chico^2/+$  (heterozygotes for the synthetic null allele),  $y\ w; chico^2/chico^2$ ,  $y\ w; chico^1/+$  (heterozygotes for the P element insertion allele),  $y\ w; chico^1/chico^1$  and  $y\ w; chico^1/chico^2$ .

#### Example 4: Clonal Analysis

- chico*<sup>1</sup> was recombined onto the FRT40 chromosome (Xu and Rubin, 1993). Germline clones of the *chico*<sup>1</sup> allele were generated using the autosomal dominant female-sterile technique in combination with the Flp recombinase system (Chou and Perrimon, 1996). Females of the genotype  $y\ w; chico^1$ , FRT40/CyO  $y^+$  were crossed with  $y\ w\ hsFlp/Y; P(ovo^{D1}\ w^+) FRT40/CyO$  males. Early third instar larvae were heat-shocked for 1.5 hr at 38°C. Females of the genotype  $y\ w\ hsFlp/y\ w; chico^1 FRT40/P(ovo^{D1}\ w^+) FRT40$  were selected and crossed to *chico*<sup>1</sup>/CyO  $y^+$  males. The resulting progeny lacking any zygotic *chico* function and their siblings bearing the CyO  $y^+$  chromosome were analyzed.

For the generation of clones in the adult eye larvae of the genotype  $y\ w\ hsFlp; chico^1 FRT40/ w^+ FRT40$

were subjected to a heat shock 24 - 48 hr AED for 1 hr at 37°C to induce mitotic recombination. Adults were examined for w clones and their corresponding twin spots (red pigmented) in the eye. Histological sections of the eyes were done as described previously (Basler and Hafen, 1988). Selective removal of *chico* function in the eye disc progenitors was achieved in animals of the genotype *y w ey-Flp; chico<sup>1</sup> FRT40 / P(w<sup>+</sup>) 1(2)2L-3.1 FRT40* and *y w ey-Flp; chico<sup>1</sup> FRT40 / P(w<sup>+</sup>) 1(2)2L-3.1 FRT40; P(w<sup>+</sup> *chico* genomic rescue construct *pCSR4*)/+.* The *eyFlp* technique has been developed by B. Dickson (personal communication).

Wing clones. Larvae of the genotype *f hsFlp/f; chico<sup>1</sup> FRT40/ck P(f<sup>+</sup>) FRT40* were subjected to a heat shock 48 - 72 hr AED for 0.5 hr at 36°C to induce mitotic recombination. Wings were mounted and examined under a compound microscope.

Disc clones. Larvae of the genotype *y w hsFlp/y w; chico<sup>1</sup> FRT40/P(arm-lacZ w<sup>+</sup>) FRT 40* and *y w hsFlp/y w; FRT40/P(arm-lacZ w<sup>+</sup>) FRT40*, respectively were subjected to a heat shock 24 - 48 hr AED for 0.5 hr at 32°C to induce mitotic recombination at a low frequency. Larvae at late third instar stage were dissected. Discs were fixed and permeabilized and stained with appropriate antibodies. Antibodies were: rabbit anti- $\beta$ -Gal (1/2000) and FITC- or TR-conjugated secondary antibodies (1/200). Actin filaments were stained using phalloidin-TR (Molecular Probes).

#### 30 **Example 5: TUNEL Assay**

Apoptotic cells were detected using the ApopTag system (ONCOR). Clones in larvae of the genotype *y w hsFlp/y w; chico<sup>1</sup> FRT40/P(arm-lacZ w<sup>+</sup>) FRT 40* and *y w hsFlp/y w; FRT40/P(arm-lacZ w<sup>+</sup>) FRT40*, respectively, and *y w hsFlp/y w; chico<sup>1</sup> FRT40/P(arm-lacZ w<sup>+</sup>) M(2L)Z FRT 40* and *y w hsFlp/y w; FRT40/P(arm-lacZ w<sup>+</sup>) M(2L)Z FRT40*, respectively, were induced as described above. Larvae at

late third instar stage were dissected. Discs were fixed and stained as described above. 3'-OH ends of DNA were labelled for 0.5 hr at 37°C by addition of digoxigenin 11-UTPs by the enzyme TdT and subsequently detected with  
5 FITC-conjugated anti-digoxigenin antibody. Discs of *GMR-grim* larvae, kindly provided by John Abrams (University of Texas), were used as positive controls.

#### Example 6: Flow Cytometry

10 Female larvae of the genotype *chico*<sup>2</sup>/+ and *chico*<sup>2</sup>/*chico*<sup>2</sup>, respectively, were dissected at late third instar stage (non-wandering stage). Dissociation of wing discs was done as described in Neufeld et al., 1998. Approximately 20 discs were dissociated. The cell  
15 suspension was analyzed using a Becton Dickinson FACStar<sup>Plus</sup> and the data was analyzed using Cell Quest (Becton Dickinson).

#### Example 7: Metabolic Studies

20 Adult males (n = 10) of the genotypes *chico*<sup>2</sup>/*chico*<sup>2</sup>, *chico*<sup>2</sup>/+ and +/+ were collected 3 days after eclosion. The fresh and dry weight, respectively, from individual males was determined. To determine the dry weight, males were fixed in 100% ethanol for 10  
25 minutes at 90°C and then dried for 2 hr at 110°C. Protein data were obtained through Kjeldahl digestion and subsequent Nesslerization (Minari and Zliversmit, 1963), with total nitrogen converted to protein by using a factor of 6.25. Glycogen and lipid data were obtained as  
30 described in van Handel and Day, 1988.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited  
35 thereto but may be otherwise variously embodied and practiced within the scope of the following claims.



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